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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Kristi D. Snell

Serial No.: 09/779,957

Art Unit: 1638

Filed: February 9, 2001

Examiner: Stuart F. Baum

For: *MULTI-GENE EXPRESSION CONSTRUCTS CONTAINING MODIFIED INTEINS*Assistant Commissioner for Patents
Washington, D.C. 20231**DECLARATION UNDER 37 C.F.R. §1.132**

Sir:

I Kristi D. Snell, hereby declare that:

1. I am the inventor of the claimed subject matter in the above-identified patent application.
2. I created DNA expression constructs expressing fusion proteins to test modified intein-mediated cleavage reactions. The DNA constructs encode green fluorescent protein (GFP) as extein 1, an intein sequence from *Pyrococcus sp.*, and beta-glucuronidase (GUS) as extein 2. Fusion expression constructs were designed for *E. coli* and plant protoplast expression studies. These fusion expression cassettes are shown in Figure 1.
3. The DNA construct for expression in *E. coli* was prepared using the method described in the paragraph bridging pages 18 and 19 of the above-identified specification. The intein used is described on page 7-9 of the specification. The N-terminus of GUS was

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engineered with an alanine to prevent normal protein splicing and to promote excision of the intein from one or more of the exteins of the fusion protein, as described on page 9, lines 12-14 of the specification. This construct was engineered with *NcoI/PstI* ends to be inserted into either an *E. coli* expression cassette or a plant expression cassette. The *E. coli* expression cassette contains a *trc* promoter and the plant expression cassette contains a C4 PPDK promoter, 35S enhancer and NOS termination sequence.

4. For *E. coli* studies, a control vector and a vector containing the fusion expression cassette of Figure 1 were transformed into the *E. coli* BL21-codon plus RP strain available from Stratagene (La Jolla, California). Starter cultures of these cells were cultured overnight at 30°C. Cells were diluted 1:100 into fresh medium and used to inoculate 100 milliliters of medium. Cells were grown for three hours at 30°C at which time 0.4 mM IPTG was added to the cultures to induce the expression of the fusion protein. Time points were analyzed at 24 hours, 31.5 hours and 46 hours for analysis by Western blot. Cells were lysed by boiling small samples in 1x gel loading buffer (New England Biolabs, Beverly, MA) without dithiothreitol. Samples of this whole extract were loaded into wells of 10-20% SDS-PAGE gradient gels. Proteins were blotted onto PVDF membranes and the membranes were probed with either anti-GFP antibodies or anti-GUS antibodies.

5. The accompanying Table 1 lists possible products associated with normal protein splicing as well as the modified intein-mediated cleavage described in this patent application. Probing Western blots with anti-GFP antibody yielded bands corresponding

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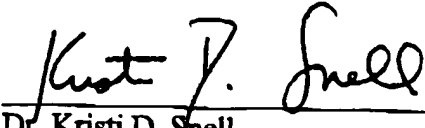
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to GFP, the linear starting material, and the branched fusion (Figure 2). No band corresponding to a spliced GFP-GUS product was observed. This blot demonstrates that the modified intein-mediated cleavage construct allows protein cleavage but prevents extein ligation. This Western blot demonstrates cleavage of the N-terminal extein (i.e. GFP) as expected. Probing blots with the anti-GUS antibody yielded bands corresponding to the linear starting material, the branched fusion, and a band at approximately 100 kDa. The 100 kDa band is most likely the intein-GUS species despite the fact that it is predicted to run at 130 kDa. The intein-GUS fusion or the GUS protein are expected products from the observed N-terminal GFP cleavage reaction.

6. These statements are evidenced in copies of my laboratory notebook pages appended to this declaration. I certify that these are true and correct copies of my laboratory notebook pages with the dates removed.

7. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 1/5/04
Dr. Kristi D. Snell

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FIGURE 1

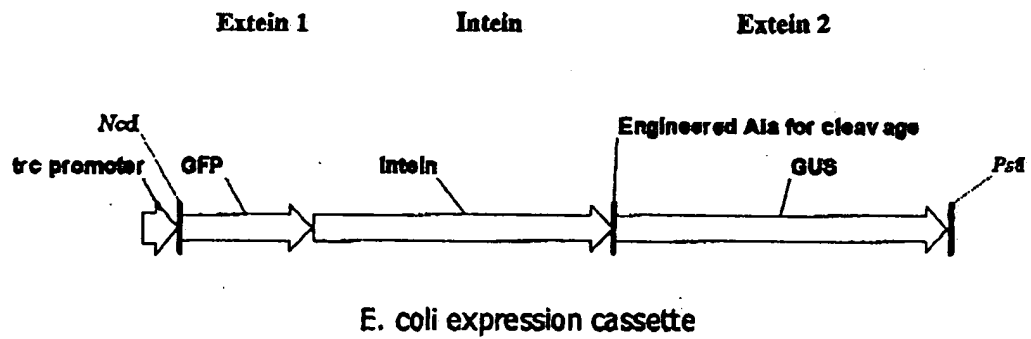
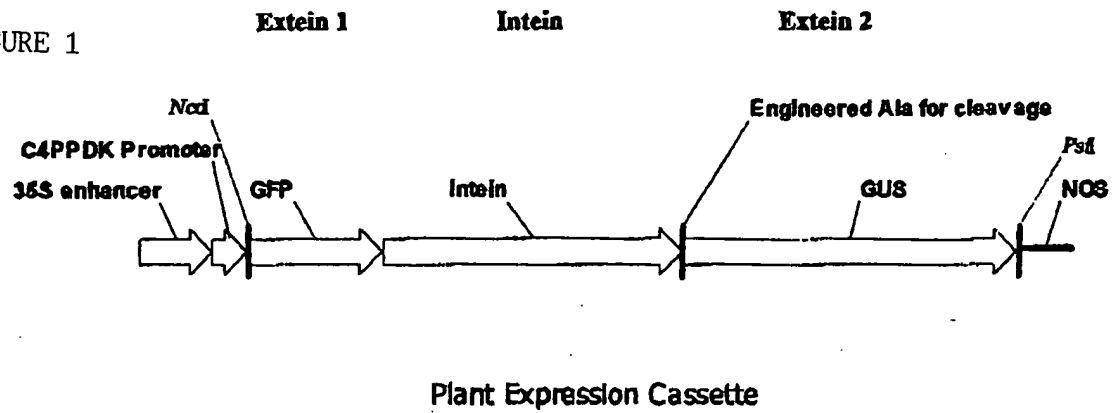


Table 1.

Possible Products of GFP-intein-GUS Cleavage Reactions


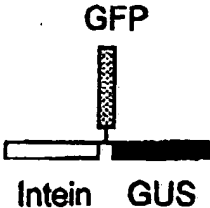




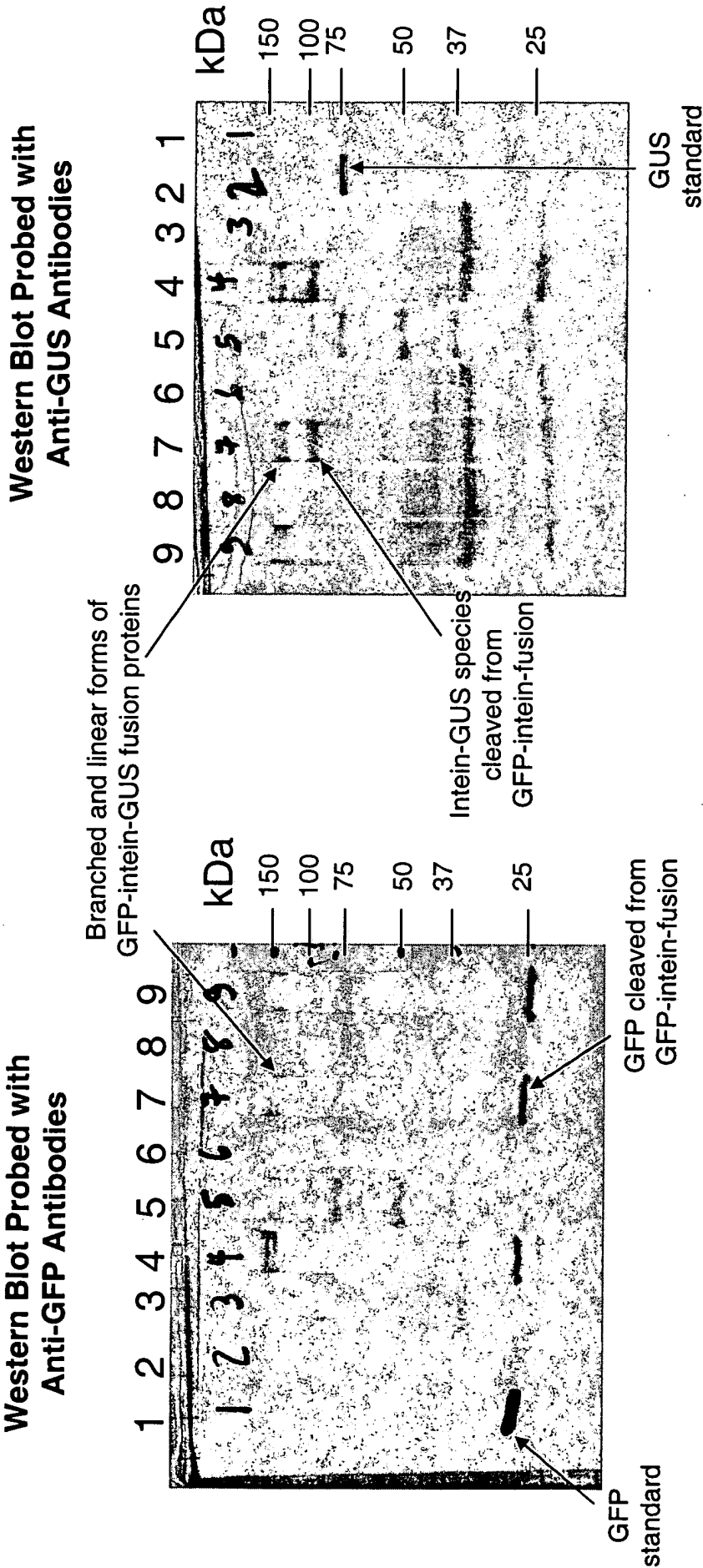
Protein Species		Expected Molecular Weight
Linear starting material		157 kDa
Branched product		≥ 157 kDa
Intein-GUS		130 kDa
GFP-GUS		95 kDa
GUS		68 kDa
GFP		26 kDa

FIGURE 2



Lane Contents	1.	2.	3.	4.	5.	6.	7.	8.	9.
	GFP standard	GUS standard	<i>E. coli</i> BL21-codon plus RP strain with vector control, 24 hour timepoint	<i>E. coli</i> BL21-codon plus RP strain with GFP-intein-Gus expression vector, 24 hour timepoint	Molecular Weight Markers	<i>E. coli</i> BL21-codon plus RP strain with vector control, 31.5 hour timepoint	<i>E. coli</i> BL21-codon plus RP strain with GFP-intein-Gus expression vector, 31.5 hour timepoint	<i>E. coli</i> BL21-codon plus RP strain with vector control, 46 hour timepoint	<i>E. coli</i> BL21-codon plus RP strain with GFP-intein-Gus expression vector, 46 hour timepoint

Expression of GFP under Gus fusion in colon plus strains

Notebook No. 25
Continued From Page 35

PROJECT

Single colonies of BL21-codon plus RP / pTRC99A,
BL21-codon plus RP / pTRC GFP under Gus; BL21-codon
plus RIL / pTRC99A, + BL21-codon plus RIL / pTRC GFP under Gus

inoculated into 5mls 2xTY Amp / Cm. Grown @
30°C overnight.

2xTY Amp / Cm in 250-ml baffled flasks.
Diluted 1:100 into 100 mls
7:50 am — 1:00 pm

Grown @ 30°C

OD₆₀₀ readings ranged from 0.883 — 1.18.

Induced w/ 0.4 ml final concentration of IPTG.
Returned to 30°C incubator.

Expt 1 Harvest 10 am W d. Frozen @ 80°C.

construct

OD₆₀₀

Amount harvested
for each of 400
cultures

Fusion in RP strain

9.114

0.439 ml

pTRC99A in RP strain

9.850

0.406 ml

Fusion in RIL strain

9.3186

0.429 ml

pTRC99A in RIL strain

10.926

0.366 ml

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Date

Timept 2

5:30pm Wed

0.7000

mLs

Harvested

tRNA in RIL strain

11.78

0.34

fusion in RIL strain

10.35

0.386

tRNA in RP strain

10.16

0.394

fusion in RP strain

10.62

0.377

Frozen @ -80°C

Timept 3

San Thoma

tRNA

0.7000

mLs harvested

fusion

8.731

0.458

tRNA

9.048

0.442

fusion

8.206

0.487

tRNA

8.858

0.453

Cells lysed in 300 μ l 1X gel loading buffer
 w/o DTT. (DTT may cause cleavage
 in vivo making it difficult to examine
 in vivo cleavage) 30 μ l of crude cell
 lysates loaded

Continued on Page

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Read and Understood By

Signed

Date

Signed

Date

PROJECT

017 452 1996 P.10/15

1 2 3 4 5 6 7 8 9 -250
-150
-100
-75
-50
-37
-25

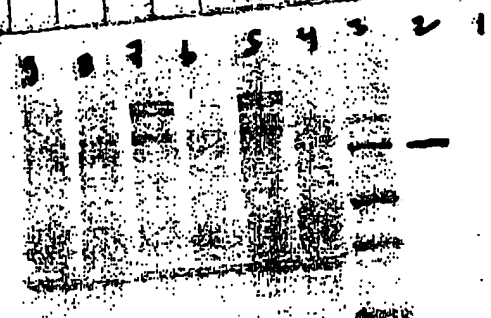
1 2 3 4 5 6 7 8 9

RP strain GFP 5 min Exposure GEL set A

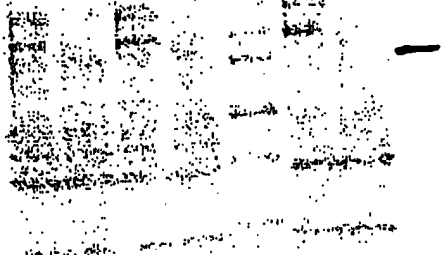
RIL strain GFP 5 min Exposure GEL set B

- P appears to have cleared from fusion well

upper bands could be branched + longer fusion NO GFP Gus fusion present



9 8 7 6 5 4 3 2 1



GEL set B Gus 14 min Exposure RIL strain

RP strain GUS 14 min exposure GEL set A

It is difficult to tell if Gus cleared well. It looks like there is a band of Gus ~100 kDa. ~~Read and Understood By~~ but this doesn't show up on GFP Western

Continued on Page

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Krista D. Snell

Signed

Date

Signed

Date

Expression of GFP in *Gus* (contn)

Notebook No.

25

OBJECT

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GELS		10-20% gels		PREDICTED PRODUCTS	
1-9	1	GFP	100 ng (10 μ L of 1/100 dil'n)	7157.9	GFP
	2	GUS	87.5 ng (10 μ L of 8.75 ng/ μ L)	157.9	GFP + GUS
	3	Tpt 1	pTRC99A in RP strain	130.6	GUS
	4	Tpt 1	fusion in RP strain	95.3	GFP + GUS
	5	MW		68.3	GUS
	6	Tpt 2	pTRC99A in RP strain	26	GFP
	7	"	fusion " " "		
	8	Tpt 3	pTRC99A in RP strain		
	9	"	fusion in " "		

This gel run 3X; 1X for staining; 1X for probing w/ GUS antibodies; 1X for probing w/ GFP antibodies.

1-9	1	GFP	100 ng (10 μ L of 1/100 dil'n)	30 μ L loaded.
	2	GUS	87.5 ng (10 μ L of 8.75 ng/ μ L)	
	3	MW		
	4	TPT 1	pTRC99A in RIL strain	
	5	TPT 1	fusion in RIL strain	
	6	TPT 2	pTRC99A in RIL strain	
	7	TPT 2	fusion in RIL strain	
	8	TPT 3	pTRC99A in RIL strain	
	9	TPT 3	fusion in RIL strain	

for staining see gels 50

This gel run 3X; 1X for staining; 1X for probing w/ GUS antibodies; 1X for probing w/ GFP antibodies.

Continued on Page

Read and Understood By

K. & T. C. 11

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